Functional Loss of the HTLV-I Tax Protein Encoded by Variant Proviruses in Infected Individuals.

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Functional Loss of the HTLV-I Tax Protein Encoded by Variant Proviruses in Infected Individuals.

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Accumulating evidence has indicated the presence of HTLV-I quasispecies in infected individuals. To elucidate their biological consequences, we amplified the whole Tax open reading frame (ORF) of HTLV-I proviruses by nested PCR from six infected individuals, including three HAM/TSP patients, and a cloned HTLV-I DNA, pMT2, and the products were introduced into an expression vector. The potential for transcriptional transactivation of protein products of independent 20-39 tax clones derived from each sample was evaluated by transfecting into pA18G-BHK-21 cells containing the HTLV-I LTR-driven lacZ gene. While all of 30 clones derived from pMT2 gave positive results, significant proportions, ranged between 16.0 and 35.0%, of the tax clones from the infected individuals were functionally defective. The functional loss of these tax clones was confirmed by chloramphenicol acetyltransferase (CAT) assay in cells cotransfected with an HTLV-I LTR-CAT reporter. DNA sequence analysis revealed that the defective clones contained at least one nonsynonymous nucleotide substitutions from the consensus sequences of the individual. These findings strongly suggested that the accumulation of HTLV-I proviruses with defective tax was a common feature among infected individuals. Since the Tax protein is indispensable for viral replication, these defective viruses were likely to be generated in individuals after the event of infection. It is conceivable that the quasispecies plays a key role in the latency of HTLV-I infection and possibly in HTLV-I-related pathogenesis.

Key words : HTLV-I, transactivator, quasispecies, defective viruses

Introduction

Human T-cell lymphotropic virus type I (HTLV-I) is a causative agent of adult T-cell leukemia (ATL), HTLV-I-associated myelopathy/tropical spastic paraparesis (HAM/TSP), and other chronic inflammatory disorders of various organs. HTLV-I transmits horizontally via sexual contact and blood transfusion as well as vertically through breastfeeding. The virus persists as a proviral DNA in T cells of infected individuals and after a long latency a minor population of carriers develops HTLV-I-associated diseases. The molecular mechanisms involved in exerting the pathogenicity of the virus are not yet fully understood.

HTLV-I is a very old virus which has been conserved among human populations in some geographical regions for a long time. In contrast to other RNA viruses including human immunodeficiency virus (HIV), HTLV-I is thought to be structurally stable because little divergence in the nucleotide sequence has been identified among HTLV-I isolates derived from different geographical regions throughout the world. The nucleotide diversity of HTLV-I among isolates is estimated to be about 20 times smaller than that of other RNA viruses such as the influenza A virus and HIV. However, recent studies have shown some intrastrain variability in the pX region of the HTLV-I proviral genome, indicating the presence of the HTLV-I quasispecies in an infected individual. The pX region encodes a transcriptional transactivator, Tax, which activates its own viral promoter in the LTR sequence and is thought to be indispensable for viral replication. In addition, Tax has the potential to upregulate the expression of various cellular genes involving cell proliferation, including interleukin-2 (IL2), IL2 receptor-α, IL6, GM-CSF, and c-fos. Moreover, Tax is known to be a dominant target antigenic molecule of cytotoxic T lymphocytes (CTL) against HTLV-I in vivo. The Tax protein is therefore considered to be a key molecule for viral replication, persistency, and the pathogenicity.
of HTLV-I.

In the present study, in order to elucidate the biological consequences of the HTLV-I quasispecies in infected individuals, we evaluated the potential for transactivation on HTLV-I LTR of the Tax protein encoded by random plasmid clones of the Tax open reading frame (ORF) derived from HTLV-I proviral DNA of six infected individuals. The results indicate that proviruses with functionally defective tax genes commonly accumulate in vivo.

MATERIALS AND METHODS

DNA preparations.

The experimental protocol was approved by the Ethics Review Committee for Human Experimentation at our institution and an informed consent was obtained from all subjects. Heparinized peripheral mononuclear cells (PBMC) and/or bronchoalveolar lavage cells (BALC) were obtained from three HAM/TSP patients and HTLV-I carriers. The PBMCs were isolated by Ficoll-Conray gradient centrifugation (Daiichi Pharmaceutica, Tokyo, Japan), as described\(^1\). Approximately 10\(^7\) cells were lysed in 0.5 ml of lysing buffer (150 mM NaCl, 10 mM Tris-HCl [pH8], 10 mM EDTA, 0.5% SDS) and treated with proteinase K (100 \(\mu\)g/ml) for 2 h at 50 °C. After phenol/chloroform extraction and ethanol precipitation, high molecular weight DNA was resuspended in 10 mM Tris (pH8), 1 mM EDTA.

Plasmids and cells

The expression vector pCG has been described and characterized elsewhere\(^2\). The pCHL4 contained the chloramphenicol acetyltransferase (CAT) gene driven by a Smal-BglI 1,630 by fragment of HTLV-I LTR\(^3\). We used the pA18G-BHK-21 cell line (a kind gift from Dr. Astier-Gin), a Syrian hamster kidney cell line stably transfected with a plasmid vector containing the lacZ gene under the control of HTLV-I LTR promoter, whose expression was inducible by the Tax protein\(^4\). Both the cell lines used (pA18G-BHK-21 and A293T) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS).

Amplification and cloning of HTLV-I tax DNA.

The whole Tax-ORF of the HTLV-I provirus was amplified by nested PCR from 0.5 \(\mu\)g of DNA in a 50 \(\mu\)l cocktail containing 10 mM Tris-HCl (pH7.5), 50 mM KCl, 1.5 mM MgCl\(_2\), 0.01% gelatin, 0.25 mM dNTPs, 1 \(\mu\)M each of primers, and 2.6 units of High Fidelity Taq Polymerase (Boehringer Mannheim). The primers used were: Tax11 (5'-GATAGCAAAACGTCAAGGCACAG-3'; positions 7158 to 7179) and Tax6 (5'-TCTGACTGTTCCACGCTTT-3'; positions 8650 to 8629) for the first amplification, and Tax15-XB (5'-TACTCTAGACCATGGCCCCACTTCC-3'; positions 7324 to 7337) and Tax30-Bam (5'-CTGAGGATCAGAGCTTAGTCT-3'; positions 8409 to 8397) for the second amplification. Xbal and BamHI restriction sites (underlined) were included in Tax15-XB and Tax30-Bam, respectively, to allow subsequent subcloning into pCG. PCR was performed in a Thermal Cycler (Biometra) for 30 cycles: denaturation at 94 °C for 30 sec (2 min on the first cycle), annealing at 60 °C for 30 sec, and extension at 72 °C for 70 sec (7 min on the last cycle). For the nested PCR, 1/10 volume of the first-step PCR product purified by Supercel II column (Takara Co, Tokyo) was further amplified for 27 cycles using the inner primer pair. The thermal cycle program was almost the same as that used for the first PCR except the annealing temperature was 58 °C for the first 6 cycles and 63 °C for the following 21 cycles. The nested PCR product was purified on a 1.0% agarose gel (Seakem GTG), digested with Xbal and BamHI, and ligated into the expression plasmid pCG. After transformation of E. Coli (DH5\(_a\)), the recombinant clones were randomly picked up and the plasmid DNA were miniprepared by the NaOH/SDS method.

Transfection.

Transient transfection was performed by the lipofection method\(^5\). Plasmid DNA mixed with 5 \(\mu\)l of Lipofectamine (GIBCO/BRL) was added to subconfluent cells cultured in 5 ml of serum-free DMEM in a 10 cm-diameter dish. After incubation for 8 h at 37 °C, 5 ml of DMEM containing 20% FBS was added, and at 24 h, the medium was replaced with DMEM containing 10% FBS. The cells were used for the following assays 48 h after transfection.

Colorimetric assay.

The pA18G-BHK-21 cells transfected with pCG-tax (0.2\(\mu\)g) were fixed with 25% glutaraldehyde and washed three times with PBS. The cells were then mixed with 0.1% X-gal solution and incubated overnight at 37 °C. When stained cells were observed in 10 view-fields under an inverse microscope (magnification: 100x), the sample scored positive for transactivation via HTLV-I LTR. Reproducibility of the negative result was confirmed by repetitive experiments.
Chlamydomenol acetyltransferase (CAT) assay.

A293T cells co-transfected with pCG-tax (0.2 \( \mu \)g) and pCHL4 (1 \( \mu \)g) were harvested and lysed 48 h after transfection. Cell extracts (50 \( \mu \)l) containing 10 \( \mu \)g of cellular proteins were mixed with 55 \( \mu \)l of 1 M Tris-HCl (pH7.8), 5 \( \mu \)l of \( ^{14} \text{C} \)-chlamydomenol (0.05 Ci/ml), and 20 \( \mu \)l of acetyl coenzyme A. After incubation for 4 h at 37 \( ^\circ \)C, chlamydomenol and its acetylated derivatives were analyzed by silica-gel thin-layer chromatography and autoradiography. The radioactivity of the spots was quantified using an image analyzer (BAS 2000; Fuji Film, Tokyo, Japan). Efficiency of each transfection was monitored by co-transfecting pRSV-lacZ.

DNA sequencing.

The whole Tax ORF (1,059 bp) in the plasmid DNA was sequenced by the dideoxy chain termination method (Thermo Sequenase core sequencing kit, Amersham) using the two kinds of 5′ Texas Red-labeled primers corresponding to the flanking vector sequences (HSV-tk sequence; 5′-GCCCATCCCTTATTGAGAA-3′, and rabbit-\( \beta \)-globin sequence; 5′-TAGCGA AAA AGAAGAC-3′), and analyzed on an automated sequencer (SQ5500; Hitachi Co, Tokyo, Japan). A sequence comparison was carried out using the DDBJ database (J02029).

RESULTS

HTLV-I proviruses with the functionally defective tax gene in infected individuals.

We analyzed six HTLV-I-infected individuals including three patients with HAM/TSP (I.T., N.O., and D.K.) and three healthy carriers (N.M., N.Y., and D.E.). In consistency with previous findings, the HAM/TSP patients had relatively higher serum antibody titers and viral loads (data not shown). The whole Tax ORF was amplified from genomic DNA samples of the infected individuals as well as plasmid DNA containing a cloned HTLV-I, pMT225. The amplified products were inserted into an expression plasmid and random 20-39 tax clones were obtained from each product. In order to define the biological consequence of in vivo HTLV-I quasispecies, the function of each of the tax clones was evaluated by transfection and a successive colorimetric assay in pA18G-BHK-21 cells with the lacZ gene under the control of the HTLV-I LTR promoter. Transfection of the wild-type tax successfully induced lacZ expression, while the expression plasmid without tax insert did not gave any stained cell (Fig. 1). When stained cells were observed in 10 view-fields under an inverse microscope, the sample scored positive for transactivation via HTLV-I LTR. As shown in Table 1,

![Fig. 1 Transcriptional transactivation of HTLV-I LTR by Tax. The function of each of the tax clones was evaluated by transfection and a successive colorimetric assay in pA18G-BHK-21 cells with the lacZ gene under the control of the HTLV-I LTR promoter. While the expression plasmid without tax insert (A) gave no stained cell, transfection of the wild-type tax (B) successfully induced lacZ expression,](image-url)
Table 1. Proportion of nonfunctional tax clones in HAM/TSP patients and healthy carriers

<table>
<thead>
<tr>
<th>Disease</th>
<th>Subjects</th>
<th>No. of total clones</th>
<th>No. of clones competent for transactivation</th>
<th>No. of clones defective for transactivation</th>
<th>Percentage of defective clones (%)</th>
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<td>pMT2</td>
<td>L.T.</td>
<td>30</td>
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<td>HAM/TSP</td>
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<td>39</td>
<td>31</td>
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<td>D.A.</td>
<td>25</td>
<td>21</td>
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<td>Healthy Carriers</td>
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<td>31</td>
<td>7</td>
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<td>N.Y.</td>
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<td>13</td>
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<td>D.H.</td>
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All of 30 clones derived from pMT2 gave positive results. On the other hand, significant proportions, ranged between 16 and 35%, of the tax clones from the infected individuals were functionally defective. Frequencies of the defective tax among three healthy carriers, 18.4, 35.0, and 32.0% (average: 29.4%), were higher than those among the HAM/TSP patients, 20.5, 16.0, and 16.0% (average: 17.5%), although the differences between the two were statistically insignificant. All the negative results were reproducible in repetitive colorimetric assays and were further confirmed by CAT assay in A293T cells cotransfected with a reporter plasmid, pCHL4. Only a representative CAT assay is shown in Fig. 2. The clones (2P10, 2B16, 3P07, 3B07, and 3P19) defective in the colorimetric assay exhibited significant CAT activity, but its level (% conversion: 3.1-8.9%) was equivalent to that in the cells transfected with the vector without tax insert (8.3%). In contrast, functionally competent clones (3B21, 2P14, 2B05, 2P08, and 3P05) were capable of converting more than 93.1% of chloramphenicol into its acetylated forms. Additional CAT assays for the remaining tax clones reproduced exclusively the results in the colorimetric assay. Expression levels of β-galactosidase encoded by the co-transfected pRSV-lacZ were roughly equivalent among each transfection (data not shown), excluding the possibility that low transfection efficiency affected the results of the reporter assay.

Functional loss of in vivo-derived tax clones as a consequence of nonsynonymous nucleotide substitution.

To analyze the sequence variation leading to functional loss of the tax gene of HTLV-I proviruses in infected individuals, 39 tax clones derived from PBMC and

Table 2. Correlation between functional loss and nonsynonymous nucleotide substitution in tax clones from an HAM/TSP patient

<table>
<thead>
<tr>
<th>Transactivation</th>
<th>No. of total clones</th>
<th>No. of clones with nonsynonymous substitution</th>
<th>No. of clones without nonsynonymous substitution</th>
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<td>(+)</td>
<td>31</td>
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BALC of a HAM patient (I.T.) were subjected to the sequence analysis. Sequence comparison found two consensus sequences (Fig. 3). One (consensus A) was identical to the reference, ATKI-36, while the other (consensus B) was different at three nucleotide positions from ATKI. Among the 39 clones analyzed, 5 and 9 exactly shared the sequence with consensus A and B, respectively, but the remaining 25 contained one to four nucleotide substitutions. The distribution of the 41 substitutions identified in these 25 clones was random and encompassed the whole region of the tax gene. About three quarters of the substitutions (31/41) resulted in amino acid changes (nonsynonymous substitution), and a half of the clones (20/39) had one or more nonsynonymous substitutions.

All the defective tax clones contained one or more nonsynonymous nucleotide substitutions (Table 2). Among the 20 clones with nonsynonymous nucleotide substitutions, 8 (40%) were functionally defective. As shown in Fig. 4, amino acid substitutions possibly causing the functional loss of the Tax protein are random and distributed throughout the Tax sequence. In one clones (2P01), nonsynonymous nucleotide substitution resulted in a termination codon at near the amino-terminal portion of the Tax. A single amino acid substitution found in a clone (G14R in 2P10) is likely to be solely responsible for the functional loss. The G14R substitution was also found in the other two defective clones (2P11 and 2B01). Although the localization was random, leucine-to-proline (L/P) and serine-to-proline (S/P) substitutions were frequent. Interestingly, all the nonsynonymous nucleotide substitutions (T-C conversion) resulting in the proline residue were always duplicated in the defective tax clones (2P11, 2B08, 2B16, and 2B17). In contrast, each of such substitutions found in three functionally competent clones was at only a single position (data not shown).

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Fig. 3 Nucleotide sequence variation among in vivo-derived HTLV-I tax clones. The whole Tax-ORF amplified from a sample DNA was ligated into pCG, and randomly selected plasmid clones were sequenced. Nucleotide sequences of 39 tax clones derived from an HTLV-I-infected individuals, I.T. are aligned in comparison with the reference ATKI-36. Dots indicate sequences identical to the reference. Italicized letters indicate nonsynonymous substitutions, and those resulting in the translational terminal codon, in particular, are underlined. The dominant sequences in the individual are indicated as consensus sequences (CONS.). The number of clones identical to a given nucleotide sequence (FREQ) and the positive (+) or negative (-) result for the colorimetric assay (COLOR. ASSAY) in transfected pA18G-BHK-21 cells are shown on the right.
Discussion

The present study has strongly suggested that a significant proportion of the tax gene of HTLV-I proviruses in infected individuals is functionally defective. DNA sequencing of the tax clones from an individual revealed that the defective clones contained at least one nonsynonymous nucleotide substitution from the consensus sequences of the individual. The Tax protein activates viral transcription through interactions with the cellular transcription factor, the cyclic AMP response element (CRE)-binding protein (CREB), and its coactivator, CREB binding protein (CBP)\(^{37,38}\). Tax is thought to stabilize CREB-DNA binding\(^{29}\) and also to serve as a bridging molecule to recruit CBP to the viral promoter\(^{40}\). Previous in vitro mutagenesis studies have revealed that nonfunctional missense mutants involve residues located throughout the linear tax sequence\(^{41,42}\). This strongly suggests that subtle alterations in the protein structure have dramatic effects on Tax function by affecting some aspects of the complex interaction with cellular proteins. The random localization of amino acid substitution in in vivo-derived mutants of nonfunctional tax clones identified in the present study is consistent with this notion. Interestingly, duplicated T-C conversions resulting in a proline residue were frequently found in these missense tax mutants. Proline is one of the aliphatic amino acids but is distinct from other members because of its aliphatic side chain bonded to both the nitrogen and the \(\alpha\)-carbon atom\(^{43}\). The resulting cyclic structure markedly influences protein architecture. It is conceivable that duplicated proline substitutions dramatically change the secondary or tertiary structure of Tax and affects its interaction with cellular proteins.

Because Tax is thought to be indispensable for HTLV-I replication in infected cells, the provirus encoding nonfunctional Tax should be transcriptionally silent and should therefore no longer be infectious. Accordingly, it is likely that such a defective virus is generated in individuals after the event of infection. Reverse transcription, an error-prone process, presumably leads to the introduction of inactivating mutations during viral replication. Recently, Saito et al.\(^{19}\) have demonstrated the sequence variation of the LTR region of HTLV-I proviruses in an infected individual at a similarly high level as the tax gene. Our preliminary study has also revealed an equivalent or higher level of variation in the env region (A.M., unpublished results). If a mutation occurs at an equivalent frequency throughout the whole HTLV-I genome, a significant number of mutants involving regions other than the tax region would also be replication defective. Moreover, the presence of proviruses lacking a long gag-pol region in ATL cells has been well documented\(^{44,45,46}\). With the frequency of proviruses with nonfunctional tax ranging from 16-35\%, the majority of HTLV-I proviruses in infected individuals might be defective. In the case of another human retrovirus, HIV, a recent report has revealed that a significant population of the tat gene encoding a HIV transactivator in infected individuals is defective and has estimated that only about 0.005\% of integrated HIV proviruses could produce the infectious virus, but that such a minute fraction is sufficient to maintain a persistent infection\(^{47}\). Presumably, this is also the case in HTLV-I, and the parental or conserved strain (the consensus strain in an individual) maintains persistency in vivo. In contrast to the intrainstrain variability, little divergence in nucleotide sequence has been identified among HTLV-I isolates, even among those derived from different geographical regions in the world\(^{48,49}\). This discrepancy could be well explained if the replication-competent strain with a highly conserved sequence alone is inheritable.

In comparison with healthy carriers, HAM/TSP

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<th>1</th>
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<tr>
<td>CONS.A</td>
<td>-------------------------------</td>
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<td>CONS.B</td>
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Fig. 4 Predicted amino acid changes in non-functional Tax proteins encoded by in vivo-derived mutants. Predicted amino acid sequences of non-functional Tax are aligned in comparison with the reference ATK1\(^{36}\). In the alignment, the one-letter code is used. Dots indicate identity to the reference sequences, and asterisks indicate in-phase termination codons. The positions of amino acid substitutions in each protein are indicated on the right.
patients revealed a smaller proportion of the defective tax clone. Although mechanisms generating the HTLV-I quasispecies in vivo remain to be elucidated, it is possible that some factors with roles in determining the outcome of HTLV-I infection might affect the tax quasispecies. One of the candidates for such a factor is the level of CTL responses in HTLV-I-infected individuals, since Tax is an immunodominant protein in those responses\(^{25,27}\).

While CTL is sometimes detectable against other viral proteins such as Gag, Pol, and Env, in most cases Tax is the only protein recognized by CTL in the PBMC. Multiple CTL peptide epitopes have been identified in the linear sequence of Tax. An anti-Tax CTL response is likely to be one of the major limiting factors for HTLV-I replication in vivo and may select escape Tax variants with altered amino acid sequences in target peptides of CTLs. It is conceivable that an accumulation of nonfunctional tax in infected individuals is a consequence of such a positive selection. Indeed, according to a recent report by Niewiesk et al.\(^{12}\), the ratio of nonsynonymous versus synonymous nucleotide substitutions in variant tax sequences is consistently higher in healthy carriers than in HAM/TSP patients. Low CTL responders would develop a high viral load with less sequence diversity, and the resulting chronic T cell activation causes tissue damages such as that seen in HAM/TSP patients\(^{49}\).

More extensive sequence comparison together with immunological analyses are under in progress in our laboratory to evaluate the proposed role for the CTL response in the outcome of HTLV-I infection and the quasispecies.

A number of literature reports have described the involvement of defective or variant viruses in the chronic infection or pathogenesis of the wild-type viruses through a modulation of their replication and immune responses in vivo. The defective genome of some RNA viruses interfering with the replication of wild-type viruses are well known as defective interfering (DI) particles\(^{40}\). The involvement of defective C-type retroviruses in the immunosuppression of infected animals has also been well documented\(^{51,52,53}\). More recent studies have revealed that naturally occurring variant sequences of hepatitis B virus or HIV efficiently interfere with the recognition of wild-type peptides by CTL, resulting in the protection of infected cells from CTL attacks\(^{14,15}\). It would be worthwhile to elucidate the role of the accumulated defective HTLV-I in the latency and pathogenicity of the virus. Tax is a multifunctional protein and considered to play an important role in activating various cellular genes involving the cell growth and inflammatory cell responses. This process involves activation of the particular transcriptional factors including NF-kB\(^{18,20,21}\) and serum response factor (SRF)\(^{21}\), which are distinct from the CREB/CREB pathway used for the HTLV-I LTR activation. It would be of particular interest to examine whether the in vivo-derived Tax variants lacking the potential to activate the viral promoter preserve the function in the transcriptional activation of cellular genes via the NF-kB or SRF pathways.

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**References**